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13. ABSTRACT (Maximum 200 words)

In work supported by the US AFOSR (grant F49620-94-1-0306) we are conducting detailed biochemical and genetic studies of three strains of *Clostridium bifermentans*, obligatory anaerobic bacteria that appear to completely degrade a variety of nitroaromatic compounds, including 2,4,6-trinitrotoluene (TNT). We are determining the optimal physiological conditions for the degradative activities of *C. bifermentans* strains; and identifying and characterizing enzymes and genes involved in the biotransformation of nitroaromatic compounds by *C. bifermentans*. In our AASERT supplemental grant (AFOSR-93-1-0464) we expanded these goals to the explosive RDX (1,3,5-triaza-1,3,5-trinitrocyclohexane). The AASERT grant funded two graduate students, who characterized the ability of *C. bifermentans* to degrade RDX (Regan, K. M., and R.L. Crawford, 1994. *Biotechnol. Kett.* 16: 1081-1086), and prepared both genomic and plasmid DNA libraries from *C. bifermentans*. This genetic work will accelerate our progress toward our goal of characterizing the genetics of TNT/RDX degradation by our clostridia (K. Diedrich, M.S. thesis, University of Idaho; in preparation).

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PROGRESS REPORT

to

US Air Force Office of Scientific Research

for

Grant No. F49620-94-1-0306

Physiology, Biochemistry, and Genetics of a Pure Culture of an
Obligatory Anaerobic Bacterium That Utilizes 2,4,-6-Trinitrotoluene (TNT)

and

Grant No. F49620-93-1-0464 (AASERT Supplement)

Biodegradation of RDX by Pure Cultures of Obligatory Anaerobic Bacteria
of the Genus *Clostridium*

Principal Investigator:
Dr. Ronald L. Crawford
Professor of Microbiology

Co-Investigator:
Dr. Don L. Crawford
Professor of Microbiology

September 1, 1996

Progress Report

Objectives:

In prior work supported by the US AFOSR (AFOSR-91-0315) our group had isolated three strains of obligatory anaerobic bacteria that appeared to completely degrade a variety of nitroaromatic compounds, including 2,4,6-trinitrotoluene (TNT). These bacteria were identified as strains of *Clostridium bifermentans*. The goal of the present project (AFOSR F49620-94-1-0306) is to conduct detailed biochemical and genetic studies of these strains; specifically, to:

1. Determine the optimal physiological conditions for *Clostridium bifermentans* strains for promoting degradation of nitroaromatic compounds.
2. Identify and characterize *Clostridium bifermentans* enzymes responsible for degradation of nitroaromatic compounds.
3. Identify and characterize genes involved in the biotransformation of nitroaromatic compounds by *Clostridium bifermentans*.

In the AASERT supplemental grant (AFOSR F49620-93-1-0464) we added other goals related to transformations of the explosive RDX (1,3,5-triaza-1,3,5-trinitrocyclohexane). Specifically, these goals were to:

1. Determine the optimal physiological conditions for *Clostridium bifermentans* strains for promoting degradation of RDX.
2. Identify and characterize *Clostridium bifermentans* enzymes responsible for degradation of RDX.
3. Identify and characterize genes involved in the biotransformation of RDX by *Clostridium bifermentans*.

Status of the effort:

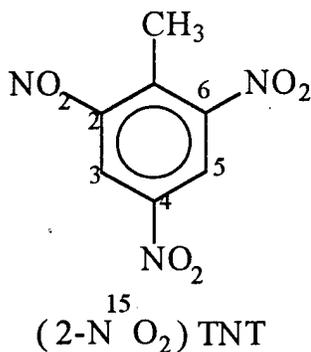
Our 1995 progress report outlined our advances toward optimizing conditions for biodegradation of 2,4,6-trinitrotoluene (TNT), 2-*sec*-butyl-4,6-dinitrophenol (dinoseb), and 1,3,5-triaza-1,3,5-trinitrocyclohexane (RDX) by *Clostridium bifermentans* strains. In continuing work during the 1996 funding period, we prepared isotopically labeled forms of TNT to use in metabolic and biodegradation pathway studies. Our labeled substrates, 2-N¹⁵O₂-TNT, 4-N¹⁵O₂-TNT, 2,4-di-N¹⁵O₂-TNT, 2,6-di-N¹⁵O₂-TNT, and 2,4,6-tri-N¹⁵O₂-TN, were used to define three types of TNT transformation activity by *C. bifermentans* LPJ1: reduction of nitro groups, hydrolysis of the reduced products to form phenols, and addition of pyruvaldehyde to reduced products to form a Schiff base. The branched pathway we proposed for catabolism of TNT by our *C. bifermentans* strains will be published shortly (Lewis, *et al.*, 1996, J. Industr. Microbiol., in press). We examined various *Clostridium* strains to establish whether TNT transformation abilities are similar in all or most clostridia, a similarity confirmed in *Clostridium bifermentans*, *acetobutylicum*, *sporogenes*, *perfringens*, and *clostridiforme* (Ederer, *et al.*, 1996, J. Industr. Microbiol., in press). We developed a reproducible method for purifying the indigenous plasmids of *C. bifermentans*, cloned a 1.6 kb fragment of one of these plasmids to begin characterizations (e.g., sequencing) that may reveal plasmid phenotypes in these organisms, and hope to develop a plasmid shuttle vector for future experiments. Finally, we prepared both genomic and plasmid DNA libraries from *C. bifermentans* TBH-1, which will accelerate progress toward our goal of characterizing the genetics of TNT/RDX degradation by our clostridia.

Accomplishments/New Findings:

1. Continuing Studies of TNT Degradation

1.1 Syntheses of labeled specimens to investigate the degradative pathway of TNT

To synthesize five possible isomers labeled in the nitro group by the ^{15}N isotope, new methods of nitration using $\text{NH}_4^{15}\text{NO}_3$ (Cambridge Isotope Laboratories, ^{15}N , 98%+) have been developed. Sulfuric acid and oleum (sulfuric acid-sulfur trioxide mixtures) were applied as proton source and dehydrating agent when one or two nitro groups were introduced into the dinitro- or mononitro-toluenes, respectively. However, when three nitro groups had to be introduced into the toluene, another approach was required. The most crucial step is the mononitration stage, when the formation of 3-nitrotoluene should be limited as much as possible. Low-temperature nitration is usually the most effective procedure, although there are some limitations, mainly the choice of solvent, to keep the reaction mixture homogenous and of low viscosity. Ammonium nitrate was found to be an extremely effective agent when used with trifluoroacetic anhydride (Crivello 1981).

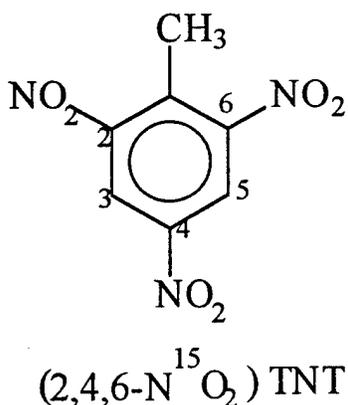


(2- $^{15}\text{NO}_2$) TNT was prepared from 2,4-dinitrotoluene in a one-step reaction using 20% oleum.

(4- $^{15}\text{NO}_2$) TNT was synthesized by using 2,6-dinitrotoluene as a substrate and 20% oleum.

(2,4-di- $^{15}\text{NO}_2$) TNT was obtained from 2-nitrotoluene using sulfuric acid and oleum (24%) in a precisely optimized time-temperature procedure.

(2,6-di- $^{15}\text{NO}_2$) TNT was synthesized from 4-nitrotoluene under precisely established time and temperature conditions and using sulfuric acid, followed by 24% oleum.



(2,4,6-tri- $^{15}\text{NO}_2$) TNT was synthesized in two stages. Using an excess amount of trifluoroacetic anhydride as reagent and solvent, toluene was introduced in one portion. The solution was cooled to -10°C and finely ground ammonium nitrate was added portionwise during stirring and cooling. The reaction mixture was then stirred at room temperature for 5 hours, put on crushed ice and extracted with ether. The extracts were washed with a 5% solution of sodium bicarbonate and dried over sodium sulfate.

After the solution was evaporated *in vacuo*, the product was taken to the second step of nitration, where it was treated with a mixture of ammonium nitrate, sulfuric acid, and 24% oleum and gradually heated to 110°C over 5 hours. The reaction mixture was then cooled and filtered. The crude product was purified by crystallization from ethanol. All samples were shown by gas chromatograph and mass spectra to be homogenous materials of high purity.

1.2 Transformation of TNT by an isolate of *C. bifermentans* from a munitions-fed bioreactor

To better understand the role of *C. bifermentans* LJP1 in a munitions-fed bioreactor and its potential for bioaugmentation, its TNT transformation activity was examined under different physiological conditions. Using cell suspensions in a buffered medium that did not support growth of the organism, we found three types of transformation activity: reduction of nitro groups, hydrolysis to yield phenolics, and adduct formation to yield a Schiff base. Reduction of nitro groups has been recognized since the initial stages of this research, which identified 4ADNT and 2,4-DANT as intermediates of TNT transformation. We assumed that the nitro groups were sequentially reduced to amino groups via nitroso and hydroxylamino intermediates to give the transient intermediates. Recent work has shown, however, that hydroxylamino intermediates such as 4HADNT, 2,4-DHANT, and 4A-2HANT accumulate to significant levels during transformation by glucose-fed cell suspensions, which indicated that reductive transformation of nitro groups proceeds by the branched pathway shown in Figure 1. The greater steady-state concentrations of DHANT indicated that reduction of hydroxylamino groups is rate-limiting on the pathway to 2,4-DANT. The ultimate product of nitro group reduction is TAT, accounting for $\approx 50\%$ of the total TNT transformation.

The balance of the TNT transformation products seen in cell suspension experiments were mostly explained by hydrolysis to yield phenolics and adduct formation to yield a Schiff base. Hydrolysis of aromatic amines may be biologically catalyzed or abiotic. TAT is readily hydrolyzed in acidic solutions, as suggested by others (Preuss *et al.* 1993) and verified by us using $H_2^{18}O$ oxygen-incorporation studies, mass spectroscopy, and NMR analysis of the major product of TAT hydrolysis. The products of TAT hydrolysis, two isomers of diaminocresol, were also seen in cell suspension experiments with TNT, although exactly how they arose is not known. Incubations of cell suspensions with TAT or 2,4-DANT yielded insignificant amounts of these products, whereas incubations with 4ADNT yielded more than those with TNT. Incubations with 2ADNT yielded a pattern of cresol isomers different than that yielded with 4ADNT or TNT. Since TAT should be much more easily hydrolyzed than the more oxidized substrates, these results are difficult to explain. Examining the aromatic amine hydrolysis activity in cell-free extracts may help us understand this phenomenon.

The third kind of activity, adduct formation, was detected by the identification of a novel Schiff base which could also be synthesized from TAT and pyruvic aldehyde (methyl glyoxal). The co-identity of these products was confirmed by mass spectroscopy using isotopically-labeled TNT and by chromatography and UV-Visible spectroscopy. Methyl glyoxal has been observed in bacterial cultures during physiologically unbalanced growth on glucose, which would indicate that it is formed by the cell suspensions and spontaneously reacts with TAT in a process not directly promoted by the organism for further metabolism or detoxification. Studies using growing cultures have not shown any net removal of the Schiff base, indicating that it is a dead-end metabolite formed only under conditions of nutrient limitation.

Comparisons of the products of ^{14}C -TNT transformation by growing cultures and cell suspensions have indicated that an additional, more polar product fraction is formed by growing cultures. It is not yet known whether the formation of this product constitutes a new type of activity or if other spontaneous chemical reactions occur with material present in complex growth media. The optimization of a method of preparing TAT from TNT should allow us to synthesize radiolabeled TAT to use in studying the mineralization of TNT metabolites by the intact consortium and by aerobic follow-up treatments.

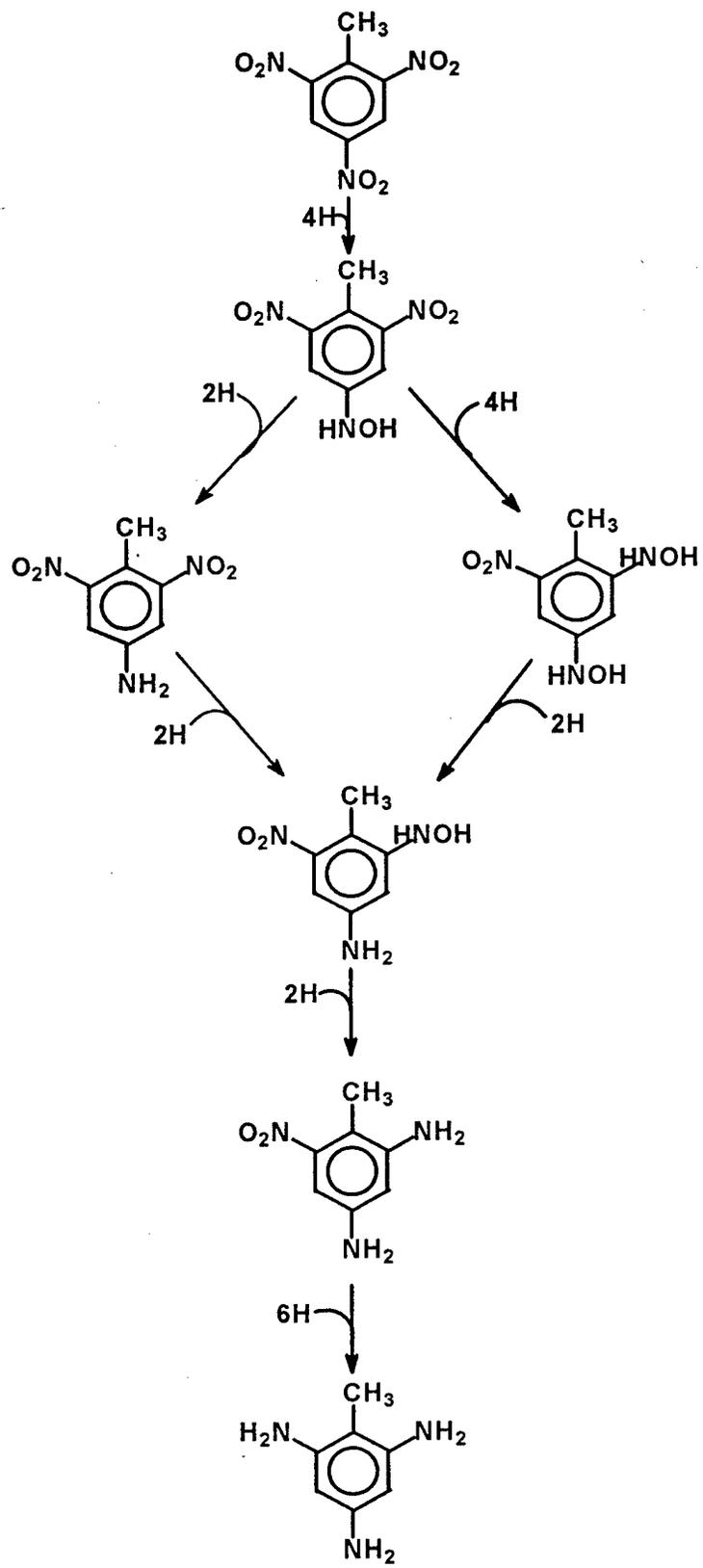


Figure 1. Reductive transformation of nitro groups.

1.3 TNT degradation by different microbial groups

In our continuing survey of TNT degradation by different clostridia, we found that in addition to the previously tested clostridia (*C. bifermentans*, *C. sordelli*, and *C. sporogenes*), three further clostridial strains obtained from ATCC (*C. perfringens*, *C. acetobutylicum*, and *C. clostridioforme*) showed comparable TNT degradation activities. All clostridia strains also seemed to accumulate comparable amounts of a novel compound, which was identified as a Schiff base of triamino-toluene and pyruvic aldehyde (Lewis *et al.*, 1996). This intermediate could not be detected during TNT degradation assays mediated by bacteria. These results indicated that the ability to degrade TNT is a general trait of clostridia. The dendrogram of the tested clostridia shown in Figure 2 is based on phylogenetic analyses by Lawson *et al.* (1993).

Since *C. bifermentans* seems to be the predominant species isolated from our munitions-fed bioreactor (four different isolates were identified as *C. bifermentans*), this strain seemed to be the species best adapted to the conditions in the bioreactor or simply the one most easily isolated by common purification techniques. To reexamine the microbial population in our bioreactor, we are planning to establish a 16S rRNA profile.

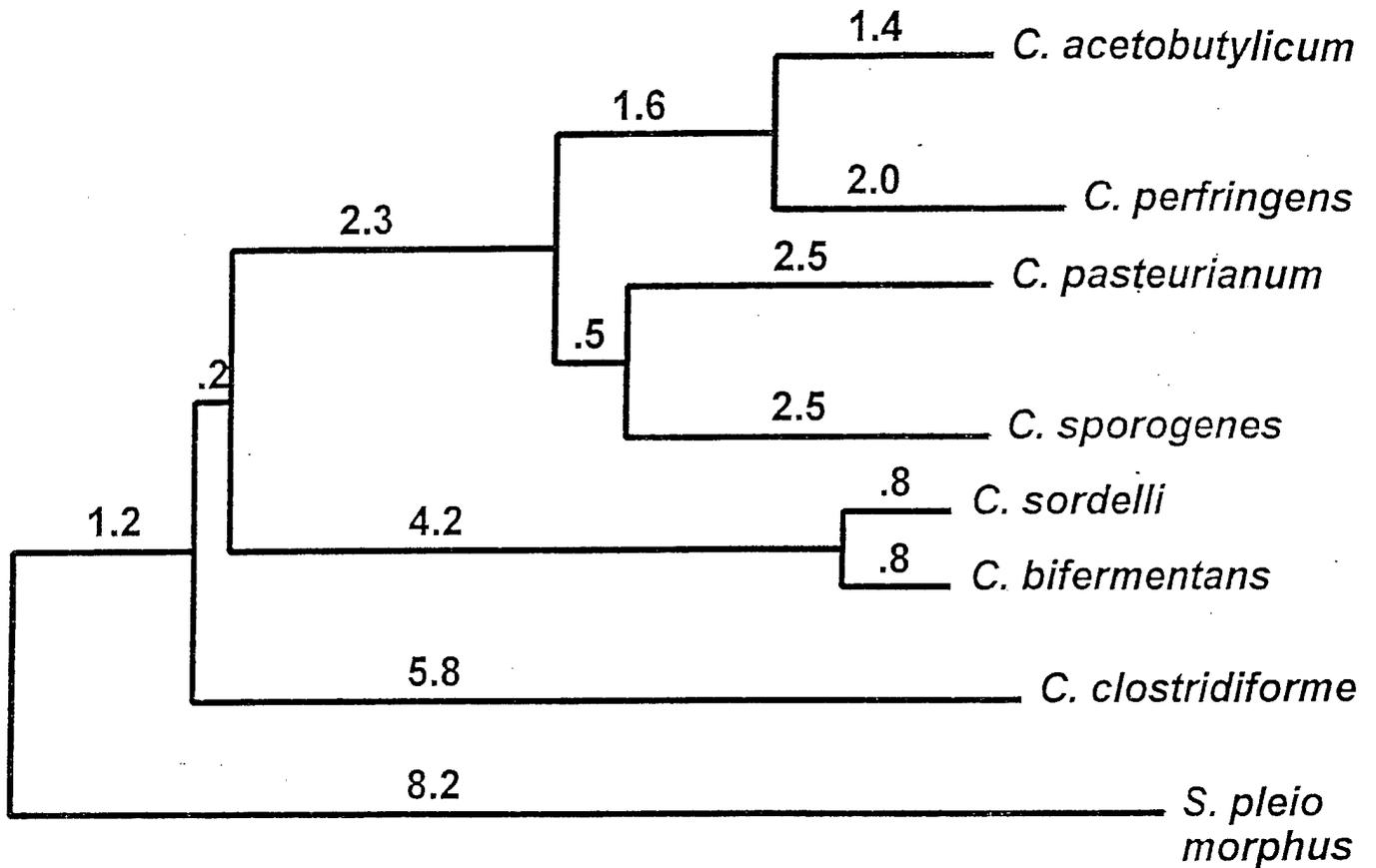
2. Genetic studies

2.1 Attempts to transform *Clostridium bifermentans*

We have not yet been able to establish a transformation system for *C. bifermentans*. We have tried a number of plasmid vectors, encoding a variety of antibiotic resistance markers with a variety of published and unpublished electroporation protocols, but we have not been able to stably introduce any of these plasmids and/or selectable markers. The compromised survival of the cells caused by oxygen exposure during the electroporation process did not seem to be the critical problem. Difficulties in the isolation of endemic *C. bifermentans* plasmid DNA indicate the presence of very active restriction system(s); however, we have not been able to identify any specific restriction activity in cell-free extracts of *C. bifermentans* degrading the DNA before it has been established.

We have several plans to address these problems:

- a. To determine whether the DNA actually enters the cell, and whether the lack of transformants is due to inoperative plasmid-encoded selectable markers and/or origin(s) of replication in *C. bifermentans*, we are planning to design PCR amplification primers for one (or more) of our available plasmids. By subjecting the "transformed" cells to DNase treatment, subsequent lysis, and PCR amplification, we should be able to observe an amplification product if the DNA does enter the cell.
- b. We are also studying the nature of the plasmids endemic to our *C. bifermentans* KMR-1 isolate, since these could become a useful tool for genetic manipulation of the organism. To determine whether this strain contains one or more plasmids, we are now cloning random *EcoR*I fragments generated from a plasmid DNA preparation of *C. bifermentans*. The fragments of one (or more) clone(s) will be used to probe total plasmid preparations and allow us to establish how many different plasmids are present in the organism. A library of plasmid DNA fragments can help identify a clostridial origin of replication and/or antibiotic resistance markers.
- c. In our current effort to characterize a plasmid from a TNT-degrading strain of *Clostridium bifermentans*, we are developing a method for purifying the plasmid, and will perform restriction analysis, hybridization studies, and sequencing so that we can eventually develop a



*Adapted from Lawson et al., 1993, FEMS microbiol. Lett. 113:87.

Fig. 2. Relationship of clostridia tested for their TNT degrading ability. Numbers above the branches represent evolutionary distance of the species. The tree is based on a Neighbor Joining analysis of 16S rRNA sequences (3).

cloning vector. A reproducible method for purifying plasmids from a gram-positive, spore-forming, anaerobic organism has been developed. Additionally, restriction analysis of a plasmid has allowed a 1.6 kb fragment to be cloned into Bluescript KS+. A probe currently being created from the fragment will be used for further hybridization studies, which will disclose the number of plasmids, as well as the similarities between multiple plasmids, if any. Further restriction mapping has also been completed. The purification of the 1.6 kb fragment could allow sequencing of this small portion of a *C. bifermentans* plasmid. After a sequence is determined, primers could be engineered to align in a reverse direction, enabling us to amplify the entire plasmid via PCR and thus advance our goal of developing a cloning vector.

- d. Since all clostridial species tested show similar TNT degradation activity, and since the mechanism of degradation seems to be very similar, if not identical, in different species of the genus, we may choose a different clostridial species as host for our genetic manipulations.

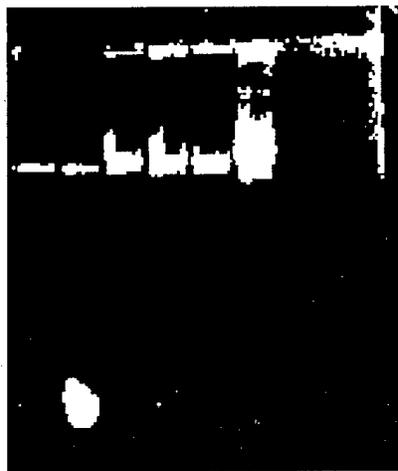
2.2 Identification of genes involved in electron transport

Genes involved in electron transport are thought to play a role in TNT degradation. Total genomic DNA digests of *C. bifermentans* were probed with a ferredoxin clone (pCP14) from *C. pasteurianum* (Graves *et al.* 1985). Even at low stringency, no distinct hybridization signal could be observed. We are planning on screening total DNA preps of the other clostridial strains available in the laboratory for the presence of loci similar to pCP14.

We are also planning to screen total DNA preps of *C. bifermentans* for the presence of sequences similar to the flavodoxin gene from *Clostridium* MP (Evan and Svenson, 1989). The genomic library that now being established will greatly aid in the cloning of the different genes.

2.3 Establishment of a DNA library

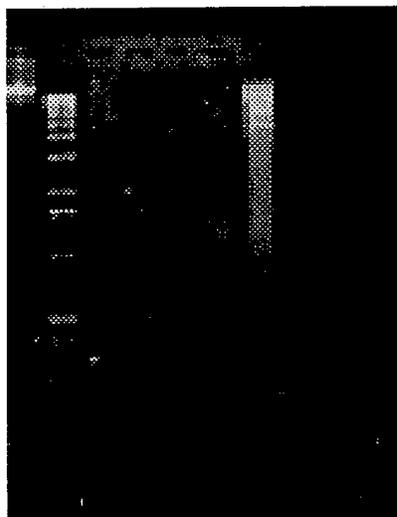
Chromosomal and plasmid DNA of *C. bifermentans* TBH-1 have been isolated, and DNA libraries created. Both the chromosomal and the plasmid DNA appear to be cross-contaminated with the other type of DNA. After *Clostridium bifermentans* TBH-1 chromosomal DNA was isolated by the procedure of Ausubel (1995), 9 μ l of each DNA sample was run on a 0.5% agarose, 0.5 mg/ml ethidium bromide gel in TAE buffer (Sambrook 1989) and visualized on a UV transilluminator (A).



A. Lanes 1, High molecular weight marker; lane 2, TBH-1 DNA without RNase A treatment; lane 3-6, TBH-1 DNA after treatment with lysozyme.

Lanes 3-6 showed bright bands migrating with the 25-48 kb marker in lane 1, but the actual size of these bands could not be resolved by agarose gel electrophoresis. A faint second band seen in lanes 3-6 migrating below the 8 kb marker, but not seen on this computer-generated image, may represent plasmid DNA.

To create the inserts for the library, the TBH-1 DNA was partially digested with *Sau3A1* and visualized by agarose gel electrophoresis. Lane 4 showed the most complete cutting (B). The DNA from lane 3 was selected for creating the library because the bulk of the DNA appeared to be near 4 kb and cutting was not as complete as in lane 4, showing that that all the *Sau3A1* sites have not been cut, so genes with *Sau3A1* sites can be included in the library.

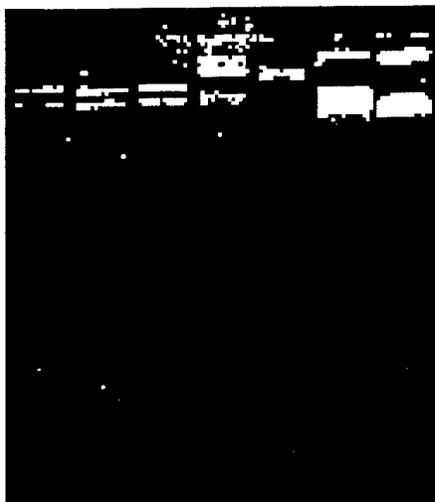


B. Lane 1, high molecular weight maker; lane 2, 1-kb ladder; lanes 3-7, DNA cut with decreasing concentrations of *Sau3A1*.

Plasmid pBR322, a 4.3-kb plasmid that fragments into 3.6-kb and 0.7-kb segments when digested with *EcoRI* and *EagI*, was isolated (Ausubel 1992) and used as the cloning vector. The results were visualized as before (C).



C. Lane 1, 1-kb ladder; lane 2, pBR322 digested with *BamHI* and treated with bacterial alkaline phosphatase; lane 3, pBR322 digested with *BamHI*; lane 4-5, pBR322.



D. Lane 1, 1-kb ladder; lanes 2-6, digested plasmid DNA from tetracycline-sensitive colonies; lane 7 pBR322 partially digested with *EcoRV* and *EagI*; lane 8, pBR322.

The vector and insert were ligated with T DNA ligase by the manufacturer's instructions (Gibco 15244-2), and ligation mixtures were dialyzed. The ligation mixtures were transformed by electroporation into *E. coli* HB101 competent cells prepared by the method of Ausubel (1992). The colonies were selected on LB plates supplemented with Carbenicillin, 100 $\mu\text{g/ml}$. The transformants were screened on LB plates with 12 $\mu\text{g/ml}$ tetracycline for inserts. Of 100 colonies screened, six grew on tetracycline plates, giving a rate of 94% inserts. Five random tetracycline sensitive colonies were selected, plasmid DNA was prepared with a Wizard Minipreps kit (Promega), and the isolated plasmid DNA was then analyzed by digestion with *EcoRV* and *EagI* (D). The average insert size was approximately 4.5 kb. A total of 10,900 transformants were collected and saved in 1-ml amounts (Ausubel 1992).

Plasmid DNA was isolated from 1 ml of the stored HB101 library by a Wizard Minipreps kit (Promega). A strong band, with smeared DNA outside, was seen migrating between the 2- and 3-kb markers (E).

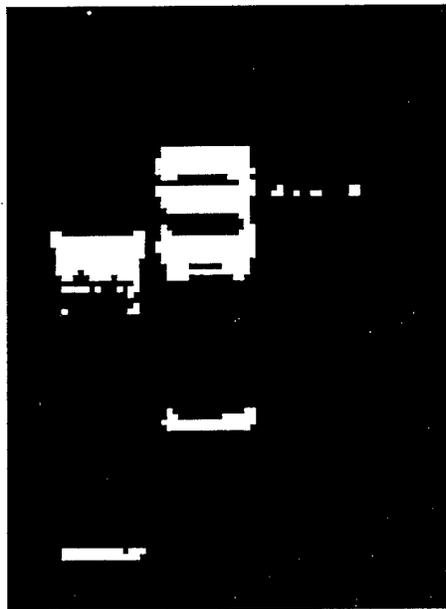


E. Lane 1, 1-kb ladder; lane 2, pBR322 plus insert.

A sample of the library DNA was digested with *EcoRV* and *EagI* and gel-analyzed. A distinct but faint band could be seen below the 4-kb marker corresponding to the larger 3.6-kb fragment of pBR322 when cut with *EcoRV* and *EagI*. Smear DNA appeared outside the band representing the smaller 0.7-kb segment of pBR322 plus inserted DNA from TBH-1.

The uncut pBR322 plus insert was dialyzed and transformed into *E. coli* F19. The transformants were selected on LB plates with 100 µg/ml carbenicillin, and screened for inserts on LB 12 µg/ml tetracycline plates. Of 100 colonies screened, two grew on tetracycline, giving 98% inserts. Five of the tetracycline-sensitive colonies were selected for Wizard Minipreps. The plasmid DNA was analyzed by digestion with *EcoRI* and *EagI*. The average insert size could not be determined since the large insert could not be discerned by agarose gel electrophoresis. Approximately 11,000 *E. coli* F19 colonies were slurried and stored in glycerol in 500 µl amounts (Ausubel 1992).

Isolation of plasmid DNA. For the isolation of *C. bifermentans* TBH-1 DNA, TBH-1 was grown overnight at 37°C in 20 ml BHI supplemented with 1.6% glycine and 30 µg/ml kanamycin. Two 3-ml portions of this overnight culture were inoculated into two anaerobically prepared 1.25 liter BHI bottles supplemented with 1.6% glycine and 30 µg/ml kanamycin, and grown to an OD₆₀₀ of 0.7. The plasmid DNA was prepared with a Qiagen Mega kit according to the manufacturer's instructions, with 400 µl TE (pH 8.0), 10 mM EDTA, and 10 mM Tris used as a resuspension media. The DNA was analyzed by agarose gel electrophoresis (F).



F. Lane 1, 1-kb ladder; lane 2, TBH-1 plasmid preparation; lane 3, high molecular weight marker.

The plasmid preparation showed strong bands at 3 kb, 8 kb and 10 kb. As in the chromosomal preparation, another strong band migrated by the 25-48 kb markers. The plasmid preparation was phenol-chloroform-extracted and ethanol-precipitated to remove nuclease contaminants that destroyed DNA preparations when the EDTA was lowered. The sample was resuspended in sterile H₂O and the DNA partially digested with *Sau3A1* (G).



G. Lane 1, 1-kb ladder; lanes 2-7, TBH-1 plasmid preparation digested with *Sau3A1*; 8, high molecular weight marker.

The sample in lane 2 was selected for use as inserts in the library, again using the pBR322 vector. After ligation with T₄ DNA ligase, the ligation mixtures were transformed into *E. coli* HB101 as before and selected on LB 100 µg/ml carbenicillin plates. Screening on LB 12 µg/ml tetracycline plates showed inserts in 83 of 100 colonies.

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Significance and Potential Applications:

Our work continues to confirm the efficacy of our patented and licensed clostridia-based systems as bioremediation processes for nitroaromatic contaminants (TNT, dinoseb, and probably most other aryl-nitro compounds) and triazine munitions residues such as RDX and HMX, which are contaminants of soils and waters at DOD and civilian locations throughout the United States and the world. The basic research we are performing shows that degradation of these types of compounds by clostridial-dominated systems should produce only innocuous products, since coupled products such as are seen in aerobic systems do not persist in the anoxic clostridial processes. Several of our pure clostridial cultures have now been patented as bioremediation inoculants, and have been licensed for industrial use to a commercial partner, the J. R Simplot Company, Boise Idaho. Our AFOSR-sponsored basic research has provided the information required to produce large quantities of stable inoculant formulations of spores of these pure strains for use in full-scale site treatment systems.

This project has become an outstanding example of taking basic research from the laboratory, through pilot testing, and into full-scale commercialization. Our continuing genetic work will lead to improvements in the overall technology, and potentially to the use of clostridia *in situ* within environments such as groundwater, where it will be important to monitor the fate of introduced microorganisms and their genes.

Personnel Supported or Associated with the Project:

I. The following individuals were supported partially or fully from AFOSR funds.

Faculty: None (University salaried employees)
Graduate Students: Mr. Karl Diedrich, M.S student, microbiology (F49620-93-1-0464)
Ms. Deborah Nichols, M.S student, microbiology
Postdoctoral: Dr. Martina Ederer, postdoctoral associate
Technical: Ms. Deanna Moser, scientific aide

II. The following individuals have been associated with the AFOSR effort, though paid on other funds.

Faculty: Dr. Ronald L. Crawford, professor of microbiology
Dr. Don L. Crawford, professor of microbiology
Dr. Roger Korus, professor of chemical engineering
Graduate Students: Mr. Steven Funk, Ph.D. student, microbiology
Mr. Terry Hammill, Ph.D. student
Postdoctoral: Dr. Stefan Goszczynski, visiting professor
Dr. Tom Lewis, postdoctoral associate

Publications (9/95-9/96):

A. Refereed Journals

1. Chin, C.-Y., T. A. Lewis, and D. L. Crawford. 1996. Reductive pathway of 2,4,6-trinitrotoluene (TNT) metabolism by *Clostridium bifermentans*. Submitted.
2. Ederer, M. M., T. A. Lewis, and R. L. Crawford. 1996. 2,4,6-trinitrotoluene (TNT) transformation by clostridia isolated from a munition-fed bioreactor: comparison with non-adapted bacteria. J. Indust. Microbiol. In press.

3. Hammill, T. B., and R. L. Crawford. 1996. Degradation of 2-sec-butyl-4,6-dinitrophenol (dinoseb) by *Clostridium bifermentans* KMR-1. *Appl. Environ. Microbiol.* 62: 1842-1846.
4. Lewis, T. A., M. M. Ederer, R. L. Crawford, and D. L. Crawford. 1996. Microbial transformation of 2,4,6-trinitrotoluene. *J. Indust. Microbiol.* In press.
5. Lewis, T. A., S. Goszczynski, R. L. Crawford, R. A. Korus, and W. Admassu. 1996. Products of anaerobic 2,4,6-trinitrotoluene (TNT) transformation by *Clostridium bifermentans*. *Appl. Environ. Microbiol.* In press.
6. Pasti-Grigsby, M. B., T. A. Lewis, D. L. Crawford, and R. L. Crawford. 1996. Transformation of 2,4,6-trinitrotoluene (TNT) by actinomycetes isolated from explosives contaminated and uncontaminated environments. *Appl. Environ. Microbiol.* 62: 1120-1123.

B. Book Chapters (9/95-9/96)

1. Funk, S. B., D. L. Crawford, and R. L. Crawford. 1996. Bioremediation of nitroaromatic explosives contaminated soils and waters. In R. L. Crawford and D. L. Crawford (ed.), *Bioremediation: principles and applications*. Cambridge University Press, United Kingdom. In press.
2. Crawford, D. L., and R. L. Crawford (ed.). *Bioremediation: principles and applications*. Cambridge University Press, Cambridge, United Kingdom. In press.

Interactions (1996):

A. Conferences:

American Society for Microbiology Annual Meeting
New Orleans, May 19-23, 1996

Papers presented:

Comparison of 2,4,6-trinitrotoluene (TNT) transformation by clostridia isolated from a munition-fed bioreactor, and by non-adapted bacteria.

Degradation of 2-sec-butyl-4,6-dinitrophenol (dinoseb) by a strain of Clostridium bifermentans.

Detection of specific microbial populations in contaminated and uncontaminated soils by multiplex PCR or denaturing gradient gel electrophoresis.

GBF-UIB Symposium on Biodegradation of Organic Pollutants
Palma, Mallorca; June 29-July 3, 1996.

Paper presented: *In situ bioremediation of dinoseb (2-sec-butyl-4,6-dinitrophenol) in groundwater*

B. Seminars presented: none

C. Review panels:

DOE Environmental Management and Sciences proposal review panel. Washington, DC, June 17-18, 1996. Contact: Dr. Jay Grimes.

EPA Bioremediation proposal review panel. Washington, DC, July 15-17, 1996. Contact: Dr. Alfred Galli.

Transitions:

The anaerobic bioremediation technology presently being researched by us under AFOSR funding has been patented, licensed, and is in full-scale commercialization by the J. R. Simplot Company, Boise, Idaho. The initial bioprocess patent (US 5,387,271) for bioremediation of soils and waters, which covers the use of all *Clostridium* spp., was supported by non-federal dollars only. This work pre-dated AFOSR funding. AFOSR funds, however, have supported much of our basic work on pure cultures that degrade nitroaromatic and nitramine compounds. A continuation in part to the original patent (Biological isolates for degrading nitroaromatics and nitramines in water and soils; US patent 5455173, October 3, 1995) did result from work supported under our AFOSR awards. The commercial rights to practice this invention (the use of these pure cultures or any similar cultures as inoculants for bioremediation purposes) are held by the J. R. Simplot Company, Boise, Idaho.

The contact at the J. R. Simplot Company concerning their commercialization of the University of Idaho technology is Mr. Gerald Mead, Vice President for Research and Product Development, PO Box 912, Pocatello, ID 83204 (208-234-5305).

The technology transfer contact at the University of Idaho is Mr. Larry Bonar, Director, Idaho Research Foundation, 121 Sweet Avenue, Moscow, Idaho 83843-2386 (208-885-3548).

New discoveries:

Biological isolates for degrading nitroaromatics and nitramines in water and soils
US patent 5455173
R. L. Crawford, D. L. Crawford, S. B. Funk, L. J. Pumfrey, K. M. Regan

Honors/Awards:

Science Team Leader, DOE NABIR Program, 1996-1999
Gamma Sigma Delta, Honor Society of Agriculture 1990-present